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Rapid molecular typing method for the reliable detection of Asiatic black truffle (*Tuber indicum*) in commercialized products: fruiting bodies and mycorrhizal seedlings

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Abstract Although authorized for the French market, the Asiatic black truffle, *Tuber indicum*, may potentially be sold as *T. melanosporum*, the "Perigord black truffle", due to difficulties in distinguishing between them by morphological criteria. A molecular method using a DNA extraction kit and specific primers based on the interspecific polymorphism of the internal transcript spacer DNA region has allowed us to amplify *T. indicum* samples (mycorrhizas, fresh, dried, and frozen ascocarps) and even to detect as little as 1% of Asiatic truffle in a mixture of ground ascocarps or mycorrhizas containing *T. melanosporum*. Our process is specific, sensitive, convenient and quick, so it could be used for the rapid detection of *T. indicum* in non-cooked food products and commercialized mycorrhizal seedlings to avoid the ecological damage which would result from its accidental introduction.

Keywords *Tuber indicum* · *Tuber melanosporum* · Internal transcript spacer sequence analysis · Polymerase chain reaction · Molecular markers

Introduction

Truffles, especially *Tuber melanosporum* Vittad., the "Perigord black truffle", are very renowned in French gastronomy due to their organoleptic qualities. They are economically important owing to the production and sale of mycorrhizal seedlings and fresh or cooked fruiting bodies (Courvoisier 1992). For the last 10 years, the truffle profession has been worrying about Asiatic truffle importation (Fourré et al. 1996), the importation of so-called Chinese truffles originating from several parts of China and probably other parts of east Asia (Mu 1998). In black truffle batches imported into Europe from China, mycologists have identified several species: *T. indicum* Cooke and Massee (1892), *T. himalayense* Zhang and Minter (1988), *T. pseudohimalayense* Moreno et al. (1997), *T. pseudoexcavatum* Wang et al. (1998), *T. sinense* Tao and Liu (1989).

Among these species, *T. indicum* is the most widespread and can be easily mistaken for *T. melanosporum*. It is also a polymorphic species and probably has several intraspecific forms (Rubini et al. 1999). At present, differentiating *T. indicum* from *T. melanosporum* under a dissecting microscope or light microscope is not always easy since the morphological characteristics of the ascocarps such as the gleba, peridium and spores (Bardet et al. 1995; Chevalier et al. 1995; Manjon et al. 1995; Di Massimo et al. 1996; Janex-Favre et al. 1996), and of the mycorrhizas, such as the cystidia and mantle (Comandini and Pacioni 1997; Gandeboeuf et al. 1997, 1998a; Zambonelli et al. 1997), are very similar between the two species. Only an experienced mycologist can distinguish one from the other thanks to the ornamentation of the spores, but when the latter are not ripe enough, it is impossible to distinguish between them.

As the price and taste of *T. indicum* are very inferior to those of *T. melanosporum*, and since the mycorrhizas and ascocarps of the two species look alike, it is very tempting to mix them up and sell the fruiting bodies or the mycorrhizal seedlings of *T. indicum* as *T. melanosporum*. Reliable identification of the truffle is therefore essential to avoid fraud and to protect the *T. melanosporum* profession. In fact, when nurserymen prepare their inoculum with ground ascocarps to make mycorrhizal seedlings (Chevalier and Grente 1979), they could unintentionally use a mixture of the two *Tuber* species. Thus,

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besides economic damage, an ecological risk is for the Perigord truffle to be eradicated by the Chinese one because in greenhouses and on plants inoculated and grown under the same conditions, *T. indicum* mycorrhizas appear faster and in larger amounts than those of *T. melanosporum* (G. Chevalier, personal communication).

Morphological similarities of the truffle species or taxa (e. g. *T. brumale* Vittad./*T. brumale* Vittad. var. *moschatum* Ferry; *T. aestivum* Vittad./*T. uncinatum* Chatin; *T. magnatum* Pico/*T. borchii* Vittad.) (Chevalier et al. 1986) that may give rise to misidentification through macroscopical or microscopical observations have made molecular approaches necessary (Lanfranco et al. 1993; Henrion et al. 1994; Amicucci et al. 1998). Molecular methods can be applied to ascocarps as well as to mycorrhizas, so it is possible to control and certify mycorrhizal seedlings at the nursery, to monitor mycorrhization in the truffiere after plantation, and to study interactions between cultivated truffle species and indigenous mycorrhizal fungi (Henrion et al. 1994).

The aim of our work was, on one hand, to develop a simple, fast, convenient and efficient DNA extraction protocol and, on the other hand, to obtain a specific and sensitive polymerase chain reaction (PCR) (Saiki et al. 1988) marker allowing the detection of *T. indicum* at low concentrations in a mixture of ground ascocarps or mycorrhizas with *T. melanosporum*. Such a marker would also detect intraspecific *T. indicum* forms independently of the physiological stage of the sample.

The marker was sought on the internal transcript spacer (ITS) which is strongly repeated and composed of two regions called ITS1 and ITS2, of nearly 150 bp each, flanking the 5.8 S ribosomal gene. It was unclear whether the high polymorphism observed on *T. indicum* ITS by different authors was related to geographical variations (Longato and Bonfante 1997; Paolocci et al. 1997) or to a misidentification of several taxa taken to be *T. indicum* (Roux et al. 1999). The interspecific variability of ITS1 and ITS2 was especially studied to establish markers for *Tuber* species (Henrion et al. 1994; Mello et al. 1996, 1999; Gandeboeuf et al. 1997; Paolocci et al. 1997, 1999).

Materials and methods

Truffle samples

Sixty-two Chinese truffles were collected in the north west and in central China from 1994 to 1999. *T. melanosporum* ascocarps were collected in central and southern France. Mycorrhizas were taken on root systems of hazel or oak seedlings inoculated with *Tuber* species according to Chevalier and Grente (1979). Samples under study were fresh, frozen, dehydrated and pickled fruiting bodies. They were identified on the basis of morphological criteria (Zhang and Minter 1988; Bardet et al. 1995; Chevalier et al. 1995; Manjon et al. 1995; Di Massimo et al. 1996; Janex-Favre et al. 1996). The samples described in Table 1 were at different physiological stages with regard to spore maturity, presence of cystidia on mycorrhizas. Furthermore, we selected *T. indicum* ascocarps with a high variability in spore size and epispore morphology, as observed by Paolocci et al. (1997) and Rubini et al. (1999).

DNA extraction

DNeasy Plant Mini Kit was used to extract DNA according to the manufacturer's instructions (Qiagen, France) with 25 mg truffles and single or pooled mycorrhizas. *T. indicum* ascocarps were mixed with *T. melanosporum* ones in percentages of: 50%, 30%, 20%, 15%, 10%, 7.5%, 5%, 2.5%, 1%, 0.5%. Four of these sporal

Table 1 isolates of *Tuber* spp. considered in this study (number of isolates used for the sequencing of its region *in parentheses*)

Species	Sample number	Collection site
Ascocarps		
Black truffles		
Chinese truffles		
T. himalayense	1	China
T. indicum	58 (3)	China
T. pseudoexcavatum	3	China
European truffles		
T. aestivum	1	France
T. brumale	11	France and Italy
T. brumale var. *moschatum*	10	France and Italy
T. melanosporum	20 (3)	France and Italy
T. mesentericum	1	France
T. uncinatum	1	France
White truffles		
T. borchii	1	Italy
T. excavatum	1	France
T. hiemalbum	1	France
T. magnatum	1	Italy
Mycorrhizas		
T. indicum	Four on *Corylus avellana*	
	Two on *Quercus pubescens*	
T. melanosporum	Three on *Corylus avellana*	
	Two on *Quercus pubescens*	

mixtures were made with different isolates clearly identified morphologically. Microscopical observations confirmed the expected spore proportions. After checking and counting *T. indicum* and *T. melanosporum* mycorrizhas under a dissecting microscope, they were mixed in the ratios: 1:1, 1:10, 1:30, 1:50, 1:75, 1:100, 1:125. A DNA control was obtained by mixing the DNA of each species in the same proportions as the sporal mixture. The DNA concentration was evaluated by spectrophotometry.

ITS amplification, purification and sequencing

The ITS1 and ITS4 primers described by White et al. (1990) were used to amplify the ITS sequences according to Henrion et al. (1994). The PCR products were purified with QIAquick PCR purification kit according to the manufacturer's protocol (Qiagen) before sequencing by the Forest Microbiology INRA unit in Nancy. The sequences have been deposited at Genbank under the accession numbers: AF300822, AF300823, AF300824 for *T. indicum* and AF300825, AF300826, AF300827 for *T. melanosporum*.

Sequence analysis

Searches for the most similar sequences in the sequence databanks were performed by the blast search algorithm (Altschul et al. 1990). Our analysis included ITS taken from the Genbank database of accession numbers: AF132501, AF167097, AF167096, U89359, Y09790 for *T. melanosporum*, and U89360, U89361, U89362, AF132502 for *T. indicum*. A similarity matrix was obtained using Old distances (Wisconsin sequence analysis, version 8; Genetic Computer Group). The sequences were aligned by ClustalW (Thompson et al. 1994) in order to obtain an ITS consensus sequence of *T. indicum* and *T. melanosporum*. The two consensus sequences were aligned with ClustalW again, in order to establish heterogeneous sites for providing putative specific *T. indicum* primers. Melting temperature, non-occurrence of secondary structures, absence of cross-hybridization and specificity of oligonucleotides were checked prior to synthesis using the PRIMER-PC software (Whitehead Institute for Biochemical Research).

Amplification using *T. indicum* primers

Two forward primers, IndF1 (5′-ACC TGT GGG AGA TCT CCA C-3′), IndF2 (5′-GGC CAT GTG TCA GAT TTA CTG-3′) and a reverse primer IndR (5′-CAT AGA CTA GCA ATT CAC TCC TG-3′) were synthesized by Oligo-Express (Paris). The reaction mix contained: 5 µl total DNA extract, 1× reaction buffer (Roche Diagnostics), 100 µM each dNTP (Pharmacia Biotech), 0.4 µM of each primer always used together as a multiplex, 0.5 units of *Taq* polymerase (Roche Diagnostics). PCR amplification was carried out in a 9600 Perkin Elmer CETUS system thermal cycler in a total volume of 25 µl. The cycling parameters were: an initial denaturation step at 94°C for 3 min; 30 cycles consisting of 30 s at 94°C, 30 s at 65°C, 30 s at 72°C; and a final extension step for 2 min at 72°C. Amplification products were separated by electrophoresis on 2% agarose gels in 1× TAE buffer (40 mM TRIS acetate, 1 mM EDTA, pH 8), stained with ethidium bromide and visualized under UV light. Positive and negative controls (no DNA template) were included in all experiments.

Results

ITS sequence analysis

The multiple sequence alignments indicated a better intraspecific variation in the ITS regions from *T. indicum*, whereas *T. melanosporum* ITS were very similar (Tables 2, 3). When comparing percentages of similarities, ITS sequences from *T. indicum* clustered into two groups called groups A and B (Table 2), which differed in length by insertions or deletions of a few base pairs on the ITS1 and ITS2 (data not shown), but various sites were equally distributed on these regions (Fig. 1). In fact, the intravariability of *T. indicum* relied on differences between ITS sequences of groups A and B, whereas a single group was quite homogeneous (Table 2).

Table 2 Similarity matrix (%) computed using Old distances (Genetic Computer Group; GCG) based on data obtained from internal transcript spacer (ITS) sequence of *T. indicum. A, B* Two different *T. indicum* groups

Genbank n°	AF300824	U89362[a]	AF300822	U89361[a]	U89360[a]	AF132502[a]	AF300823	
AF300824	100.0	99.5	97.3	97.8	92.8	93.3	92.4	B
U89362[a]		100.0	97.4	98.0	93.0	93.5	92.6	B
AF300822			100.0	99.2	92.3	92.8	91.9	B
U89361[a]				100.0	92.8	93.3	92.4	B
U89360[a]					100.0	99.5	98.7	A
AF132502[a]						100.0	99.2	A
AF300823							100.0	A

[a] Sequences obtained from Genbank; the others were sequenced in this study

Table 3 Similarity matrix (%) computed using Old distances (GCG) based on data obtained from ITS sequence of *T. melanosporum*

Genbank n°	AF167096[a]	U89359[a]	AF300826	AF132501[a]	AF167097[a]	AF300827	AF300825
AF167096[a]	100.0	99.7	99.2	99.0	98.5	99.2	99.2
U89359[a]		100.0	99.4	99.2	99.4	98.7	99.4
AF300826			100.0	99.7	99.5	99.2	99.0
AF132501[a]				100.0	99.4	99.0	98.9
AF167097[a]					100.0	98.9	98.9
AF300827						100.0	98.4
AF300825							100.0

[a] Sequences obtained from Genbank; the others were sequenced in this study

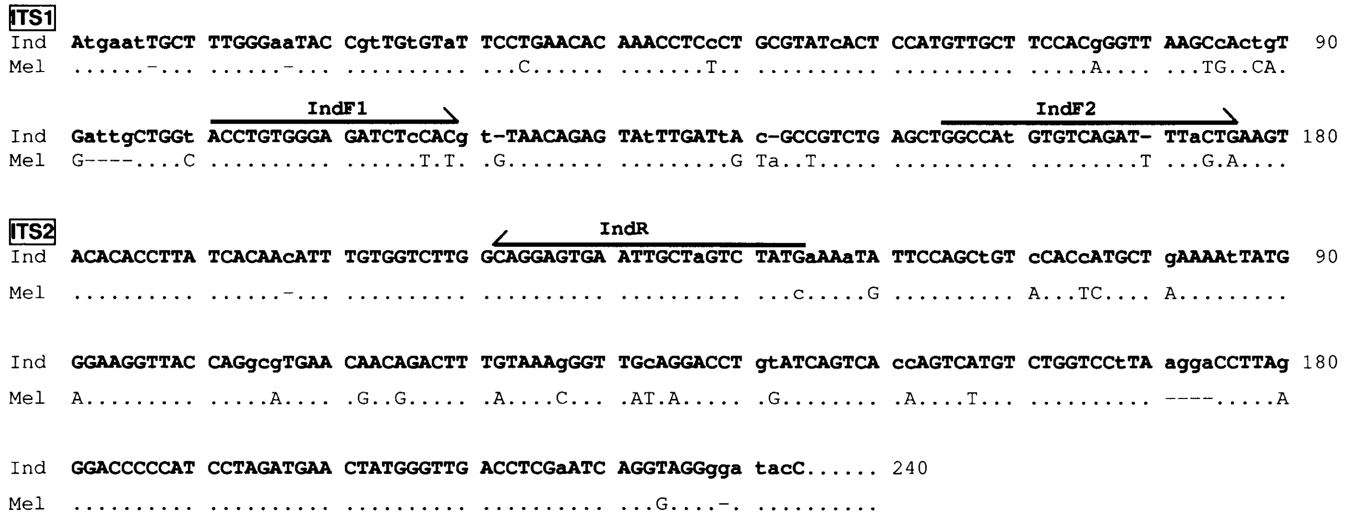

Fig. 1 Differences in ITS1 and ITS2 consensus sequences between *Tuber indicum* and *T. melanosporum*. For *T. indicum*: base identical (*dot*), indel (*dash*), conserved bases (*upper case*), variable bases (*lower case*), *T. indicum*-specific primer sequences (*arrows*)

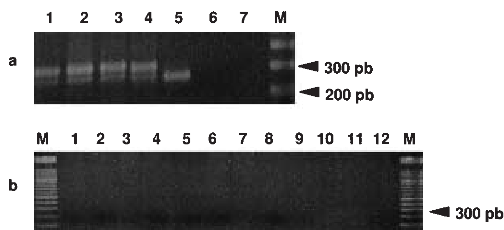

Fig. 2a, b Polymerase chain reaction (PCR) amplification using the multiplex IndF1, IndF2 and IndR with DNA from different *Tuber* species. **a** Amplification with DNA from *T. indicum* mycorrhizas and ascocarps in different physiological stages; immature ascocarp with no spores (*lane 1*), immature ascocarp with translucent spores (*lane 2*), mature ascocarp with well-ornamented spores (*lane 3*), mycorrhiza with cystidia (*lane 4*), mycorrhiza without cystidia (*lane 5*), roots without mycorrhizas (*lane 6*), no DNA template (*lane 7*), 100-bp ladder (*lane M*). **b** PCR with DNA from different *Tuber* species without amplification; black truffles T. *himalayense* (*lane 1*), *T. pseudoexcavatum* (*lane 2*), *T. aestivum* (*lane 3*), *T. brumale* (*lane 4*), *T. melanosporum* (*lane 5*), *T. mesentericum* (*lane 6*), *T. uncinatum* (*lane 7*); white truffles *T. borchii* (*lane 8*), *T. excavatum* (*lane 9*), *T. hiemalbum* (*lane 10*), *T. magnatum* (*lane 11*); no DNA template (*lane 12*), 100-bp ladder (*lane M*)

Definition of *T. indicum* primers

Alignment of the ITS consensus of *T. melanosporum* and *T. indicum* showed 88% of identity arising from single-base substitution and insertion in the ITS1 and ITS2 regions (Fig. 1). High intraspecific variation in *T. indicum* ITS and strong homology between the two *Tuber* species led us to choose three *T. indicum* primers: the two forward primers are specific but variable among *T. indicum* isolates while the reverse is on a consensus site shared with *T. melanosporum*. Therefore, when using the set of primers together as a multiplex, only *T. indicum* isolates would be amplified through PCR. Blast found no significant homologies between our primers and sequences in the database, especially with regard to ITS from other *Tuber* species except from *T. indicum*.

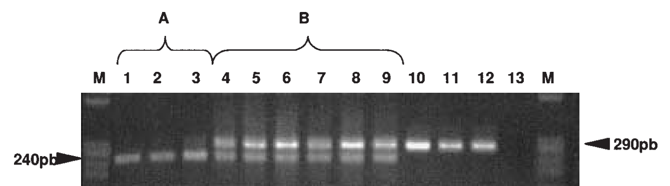

Fig. 3 An example of the three typical patterns obtained after PCR amplification of the DNA internal transcript spacer sequences extracted from 11 *T. indicum* isolates out of 50 tested with the multiplex IndF1, IndF2 and IndR. According to the isolate, amplification occurred with IndF2/IndR (*lanes 1–3*), with IndF1/IndR and IndF2/IndR (lanes *4–9*), or with IndF1/IndR (lanes *10–12*). No DNA template (*lane 13*); phiX174 DNA *Hae*III digest (*lane M*). *A, B* represent two different *T. indicum* groups

Validation of *T. indicum* marker

All *T. indicum* DNA samples displayed a single band at 240 bp, whereas no amplification occurred with the DNA of *T. melanosporum* and other *Tuber* species (Fig. 2). Three distinct patterns were exhibited (Fig. 3) corresponding to the following: one band at 240 bp amplified by the primer pair IndF2/IndR for 11 samples; another band at 290 bp amplified by IndF1/IndR for three isolates; and the same two bands amplified together by the above-mentioned primer pairs for 50 samples. *T. indicum* isolates showing the first two patterns were members respectively of groups A and B. Isolates showing the third pattern could not be placed in any group because their ITS had not been sequenced.

T. indicum band(s) were clearly visible even at 1% (Fig. 4) for overall mixtures either with ascocarps or mycorrhizas. This result was confirmed in terms of the presence and intensity of the amplicon by obtaining the same pattern with DNA mixtures, for which a 1% sample corresponded to DNA of 0.08 ng and 7.92 ng, respectively, for *T. indicum* and *T. melanosporum*.

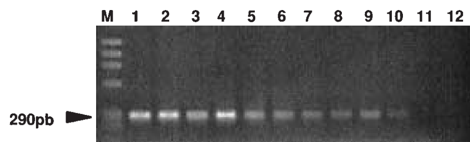

Fig. 4 PCR amplification products with DNA from sporal mixtures of *T. indicum* and *T. melanosporum* using the multiplex IndF1, IndF2 and IndR. *T. indicum* alone (*lane 1*); *T. indicum* with *T. melanosporum* in percentages of: 50% (*lane 2*), 30% (*lane 3*), 20% (*lane 4*), 15% (*lane 5*), 10% (*lane 6*), 7.5% (*lane 7*), 5% (*lane 8*), 2.5% (*lane 9*), 1% (*lane 10*), 0.5% (*lane 11*); *T. melanosporum* alone (*lane 12*); phiX174 DNA *Hae*III digest (*lane M*). Similar patterns were obtained with mycorrhizal and DNA mixtures

Discussion

The specificity and reliability of our PCR primers for *T. indicum* were displayed by DNA amplifications of a number of *T. indicum* isolates, on one hand, and by the absence of amplification of the DNA of other *Tuber* species such as *T. melanosporum,* on the other. Furthermore, the PCR reaction depended neither on the physiological stage of the mycorrhiza or fruiting body nor on the conservation of ascocarps.

Previously in a *T. indicum* and *T. melanosporum* mixture of mycorrhizas, a SCAR for *T. melanosporum* (Gandeboeuf et al. 1998b) was able to detect the Perigord truffle at a ratio of 1:20, while a SCAR for *T. indicum* (Paolocci 1999) presented the band of Chinese truffle at a ratio of 1:50. In view of these results, our marker appears to be very sensitive since *T. indicum* could still be detected at a ratio of 1:100. No test concerning the detection limit in sporal mixtures of *Tuber* spp. with molecular markers has been reported to date.

The technical approaches reported to date, such as tissue and gel analysis, are considered to be fast and easy to perform. We were able to test about 30 samples in 1 day; no band indicated the absence of *T. indicum* while one or two bands revealed the presence of the Chinese truffle. We think that the control of the truffle market and mycorrhizal seedlings would be more efficient if this biomolecular test were to be used instead of visual approaches. In fact, even when spore maturity makes it possible to distinguish *T. indicum* from other species in a mixture, the high variability in spore morphology among isolates makes its detection very tricky.

We defined a new molecular strategy based on multiplex PCR for *T. indicum* because intravariability in ITS1 and ITS2 sequences increases the probability of a PCR when using two *T. indicum* specific primers on these regions instead of one, as with the SCARs reported earlier (Paolocci et al. 1997, 1999; Séjalon-Delmas et al. 2000).

Alignments showed two groups of *T. indicum* isolates but no correlation between spore morphology and these groups, as observed by Rubini et al. (1999). Two out of three patterns displayed by our marker were common to groups A and B. For the other one, ITS from corresponding isolates could be sequenced in order to categorize them. Regarding the location of ITS from Genbank (U89360, U89361, U89362), groups A and B are similar to those described by Paolocci et al. (1997), who proposed different geographical origins for each one, while sequences in group B (U89361, U89362) could be distinct species like *T. himalayense* and/or *T. pseudohimalayense* as suggested by Roux et al. (1999). This suggests that group A could be *T. indicum* and group B the other species. However, our samples in group B exhibited typical spores of *T. indicum* and not at all typical of those of the species given above, so we support Paolocci's conclusion.

This ambiguous situation might be related to the lack of knowledge about the real origin of *T. indicum* isolates, as well as to the lack of comparison between a large number of ITS sequences in members of group B and in *T. himalayense* and/or *T. pseudohimalayense*. *T. indicum* shows so many intraspecific forms, which is related to its high morphological and molecular variability, that several species of Chinese or Asian truffles (like *T. himalayense* or *T. pseudohimalayense*) may in fact be different forms of *T. indicum*. Some taxa were probably described as a species too early, perhaps after a single sample was analysed.

Phylogenetic investigations would make it possible to study both *T. indicum* intravariability, especially with ITS from isolates showing patterns different to those of groups A and B, and relationships between *T. melanosporum* and *T. indicum*. In fact, they probably share a common origin, as suggested in a recent molecular study on two repeated *T. melanosporum* sequences (Paolocci et al. 2000).

In conclusion, compared with techniques reported so far (Paolocci et al. 1999; Séjalon-Delmas et al. 2000), our process offers total reproducibility from one laboratory to another thanks to the use of a DNA extraction kit, and the possibility of automating all the steps when analysing many samples. It is also very reliable since the method was validated on a wide range of material and took *T. indicum* ITS variability into account thanks to an original set of primers.

The method is very suitable for the large-scale control of non-transformed products and mycorrhizal seedlings in order to enforce legislation concerning the trade of truffle-based products, and to avoid ecological damage related to an accidental introduction of the Chinese truffle.

It would now be interesting to do the same study on canned truffles, although it must be realized that the limiting factors for the DNA analysis of cooked products are associated with the extraction and the choice of marker (Meyer 1999; Séjalon-Delmas et al. 2000).

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